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Noninvasive Diagnostics for Early Detection of Lung Cancer: Challenges and Potential with a Focus on Changes in DNA Methylation

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ABSTRACT

Lung cancer remains the leading cause of cancer deaths in the United States and the world. Early detection of this disease can reduce mortality, as demonstrated for low-dose computed tomography (LDCT) screening. However, there remains a need for improvements in lung cancer detection to complement LDCT screening and to increase adoption of screening. Molecular changes in the tumor, and the patient's response to the presence of the tumor, have been examined as potential biomarkers for diagnosing lung cancer. There are significant challenges to developing an effective biomarker with sufficient sensitivity and specificity for the early detection of lung cancer, particularly the detection of circulating tumor DNA, which is present in very small quantities. We will review approaches to develop biomarkers for the early detection of lung cancer, with special consideration to detection of rare tumor events, focus on the use of DNA methylation-based detection in plasma and sputum, and discuss the promise and challenges of lung cancer early detection. Plasma-based detection of lung cancer DNA methylation may provide a simple cost-effective method for the early detection of lung cancer.

See all articles in this *CEBP Focus* section, "NCI Early Detection Research Network: Making Cancer Detection Possible."

Introduction

Where we stand in lung cancer screening and diagnostics

Lung cancer is the leading cause of cancer-related mortality. The American Cancer Society estimates 228,820 new cases of lung cancer and 135,720 deaths from lung cancer in 2020. As for most adult carcinomas, screening to detect early disease before the development of symptoms allows curative approaches that significantly improve patient outcomes. The National Lung Cancer Screening Trial documented a 15% to 20% decrease in mortality when screening high-risk groups with low-dose computed tomography (LDCT) when compared with chest X-rays (1). As of 2014, the U.S. Preventive Services Task Force recommended LDCT as a routine screening tool in high-risk groups (ages 55–74, >30 pack-year smoking history, and if no longer smoking, have quit within 15 years). Additional studies, including the NELSON trial (2), have also demonstrated improvement in lung cancer mortality with LDCT screening. Although LDCT has a high sensitivity, it has a high false discovery rate, and many nodules are labeled as indeterminate risk (varying in size between 7 and 29 mm). Of the 24.2% of people who had "positive" test, 96.4% had benign lung nodules (3, 4). Limited diagnostic accuracy is achieved with LDCT, and of the lung nodules labeled as "indeterminate," only 1.7% to 22% are malignant (1). The high false-positive rate can cause unwarranted anxiety, unnecessary radiation exposure, and further invasive testing such as bronchoscopy to definitively rule out lung cancer. Obtaining a lung biopsy for confirmatory testing has risks including hemorrhage, pneumothorax, infection, and death (5–7). Current screening criteria with LDCT-based screening only accounts for one third of lung cancer patients. Of the patients who successfully undergo treatment for lung cancer, 30% of patients have tumor recurrence and eventually die from lung cancer (8). Improvements in the current lung cancer screening paradigm are needed, and noninvasive biomarkers could be an ideal complement with LDCT to improve sensitivity and specificity and decrease the rate of false positives. Therefore, developing new biomarkers for earlier disease detection, monitoring, and prognosis is necessary. Noninvasive biomarkers can also be useful for treatment monitoring, prognostication, and monitoring for disease relapse.

Potential Analytes as Biomarkers for Lung Cancer Detection

Lung cancer is associated with specific molecular changes in the tumor cell of origin as well as changes in the host that are potentially useful for lung cancer screening. Various tumor-associated molecular changes to diagnose early-stage lung cancer have been the subject of long-standing investigations and will not be completely described, as this has been the subject of previous reviews. However, some discussion is useful for context and to consider the challenges that each potential analyte introduces. Fundamental to the development of cancer are changes in DNA, including mutational and copy-number changes. These include both driver mutations that have a significant role in regulating tumor biology and passenger mutations that are phenotypically silent, and are frequent in lung cancer. Mutation rate is also accelerated secondary to the field effect from smoking, the primary risk factor for lung cancer. These changes will be further considered in later descriptions of mutational detection in circulating tumor DNA (ctDNA). Changes in DNA methylation share some of these features and will also be discussed. However, mutational and epigenetic changes in DNA result in changes in messenger RNA (mRNA), microRNA (miRNA; noncoding RNA that regulates gene expression), and proteins. Each of these has been examined for lung cancer detection. mRNA detection is challenging given the instability of mRNA due to presence of RNase in circulation and has made

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miRNA much more promising for the early detection of lung cancer. Indeed, promising studies have been published using this approach (9). Proteins, as the final product of gene expression, are also potential biomarkers for the molecular changes associated with the development of cancer. However, most protein biomarkers examined for early detection in blood are not related to mutational or expression changes within the tumor, but are rather tissue products with higher levels in serum (CEA and PSA) or related to host response (IL6 and prolactin). As such, these protein markers require quantitative cutoffs to achieve specificity, which limits the sensitivity in very early-stage cancers. Lung cancer–specific changes in levels of some proteins such as for Cytokeratin 19 (CYFRA 21–1) have provided one approach to increasing sensitivity (10). In addition, the patient response has also been the source for lung cancer detection through expression profiles of whole blood (11) and autoantibodies (12).

Liquid biopsies and ctDNA biology

Nucleic acids were first reported in blood in 1948 (13). In 1977, cancer patients reportedly had more cell-free DNA (cfDNA) than healthy volunteers (14). Since then, cfDNA levels have also been described to be increased in patients who had undergone trauma, transplant, infection, and infarctions. In 1989, Stroun and colleagues demonstrated that some of cfDNA was of tumor origin and Sidransky and colleagues showed that urine pellets from patients with urothelial cancers had TP53 mutations (15, 16).

In oncology, liquid biopsy is used to describe genomic information obtained noninvasively that would typically only be accessible by sampling a tissue biopsy. Tumor cells release ctDNA, circulating tumor RNA (miRNA, mRNA), proteins, metabolites, tumor-educated platelets, circulating tumor cells, and extracellular vesicles including exosomes (17–22). cfDNA consists of DNA fragments that are thought to be wrapped around nucleosomes and undergo degradation at sites most susceptible to DNase digestion. Thereby, these fragments have a modal length of 166 bp (23). Most of cfDNA is derived from leukocytes; however, in cancer patients, a fraction of cfDNA consists of ctDNA. There are multiple mechanisms postulated for release of ctDNA by tumor cells: apoptosis, necrosis, and active transport of vesicles (18). The amount of ctDNA in biofluid is a function of tumor size, vascular/lymphatic invasiveness, number of metastatic sites, and cancer type (24). A liquid biopsy gives a real-time genomic snapshot of the tumor as all tumor cells from all tumor sites will release DNA to some degree. Therefore, liquid biopsies manage to capture intra- and intertumoral heterogeneity that tissue biopsies cannot capture due to sampling bias, operator dependency, and multiple locations in metastatic tumors (25, 26). Moreover, repeating a noninvasive biopsy for disease monitoring entails repeating only sampling of the biofluid, whereas repeating tissue biopsies is associated with additional risks.

Clinical Applications of Liquid Biopsy

A liquid biopsy–based assay is currently established for noninvasive genotyping of ctDNA. There is potential utility in primary diagnosis and augmenting current screening tests. A noninvasive test can be potentially used to longitudinally follow-up subjects with indeterminate nodules in lung cancer without the risks of radiation exposure from repeated imaging. Noninvasive biomarkers can also prognosticate disease (27–31), and therefore prioritize the type of therapy that would be most efficacious. ctDNA levels can be followed longitudinally to monitor therapy (27, 32–35). When treatment fails, there is an increase in ctDNA signal indicative of increasing tumor burden; deeper sequencing of ctDNA in the most proximal biofluid can potentially circumvent the need for a second biopsy to guide further therapeutic options (33, 36, 37). Another application is minimal residual disease monitoring for a patient who has undergone neoadjuvant therapy (38), surgery (38, 39), or adjuvant therapy/radiation (39).

Current Landscape for Noninvasive Biomarkers

ctDNA-based assays can detect mutations, copy-number, and methylation alterations. A 58-gene targeted sequencing approach using somatic mutations by Phallen and colleagues to detect stage I or II colorectal, breast, lung, or ovarian cancer from plasma resulted in detection rate of 71%, 59%, 59%, and 68%, respectively (29). miRNA are small, noncoding RNA fragments that can regulate protein expression and are dysregulated in cancer. In order to mitigate the high rate of false positive when screening high-risk individuals for lung cancer with LDCT, the investigators used a 13-plex miRNA test on subjects enrolled in the Continuous Observation of Smoking Subjects (COSMOS) where they found comparable sensitivity and negative predictive value to LDCT (40). For stage I non–small cell lung carcinomas (NSCLC; $N = 22$), they reported a ROC curve AUC of 0.89, sensitivity 59%, and specificity 90%. The randomized Multicenter Italian Lung Detection (MILD) trial used a miRNA signature to further risk stratify subjects once they had undergone LDCT (9). These studies demonstrate that liquid biopsy–based assays can potentially be used as a screening tool and as a complement to current screening diagnostics.

DNA methylation biology

Epigenetic changes are characteristic during initiation and progression of malignancy. DNA methylation–based biomarkers are an ideal candidate because epigenetic changes occur early in carcinogenesis and ctDNA is a stable molecule in bodily fluids. DNA methylation occurs on C5 of cytosine in the CpG dinucleotide catalyzed by DNA methyltransferase. Most CpG sites are methylated in normal cells (41). However, CpG islands (density of ~1 CpG/10 bp) occur in 2% of the genome and are mostly unmethylated in normal cells (42). CpG islands (CGI) occur at transcription start sites within promoter regions and regulate gene expression. When methylation occurs in the gene promoter regions, it represses gene expression (43), and can also occur at CpG shores, shelves, and enhancer regions. Focal hypermethylation changes can drive inactivation of key tumor suppressor genes, dysregulation of regulatory regions that control cell cycle and growth, or reduced response to therapy. Global hypomethylation is considered carcinogenic on its own and can play a role in destabilizing the genetic material. The widespread nature of epigenetic changes provides multiple targets that can concomitantly be interrogated. In lung cancer carcinogenesis, alterations in methylation pattern occur early and can drive cancer progression. Lung cancer genome is universally hypomethylated except for CpG dense regions at promoter sites of tumor suppressor genes and other genes that prevent cancer progression. In NSCLC, alterations in methylation are associated with cigarette use (44), cancer screening, tumor progression (45), prognostication (46–49), histologic, and molecular classification (50–54).

There are some potential advantages for DNA methylation versus mutational detection. First, oncogenic mutations including *KRAS* and *EGFR* are typically heterozygous (no loss of wild-type allele), while methylation events, typically functioning as tumor suppressor with loss of expression, are either homozygous (with allelic loss of other

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copy) or biallelic. Second, increased apoptosis, a feature of cancer due to cellular turnover, results in an increase in circulating nucleosomes, the predominant source of ctDNA (55, 56). We and others have observed that hypermethylated promoter regions are associated with repressive chromatin-associated histone modifications and closely spaced nucleosomes (57), in contrast to the nucleosome-free region of unmethylated and actively transcribed promoters. This provides a relative enrichment for hypermethylated promoter regions through enhanced protection from nucleosomal degradation during apoptosis.

DNA methylation occurs early in lung cancer

It is difficult to determine temporality between methylation changes in DNA and carcinogenensis due to logistical limitations such as lack of adequate sampling of normal tissues before carcinogenesis. It was first shown that gene promoter hypermethylation preceded squamous cell carcinoma (SCC) lung cancer when a study utilized a 2-stage nested PCR to detect *MGMT* and *CDKN2A* promoter methylation. They detected *MGMT* and/or *CDKN2A* hypermethylation in 100% of sputum samples in subjects who were eventually diagnosed with SCC lung cancer with up to 3 years of lead time to clinical diagnosis (58). Ensuing studies have demonstrated similar results (59–62). In one study, of the 8 subjects positive for *CDKN2A* hypermethylation in sputum, 3 developed lung cancer within 1 year after sample collection (59). Similarly in another study, of the 5 subjects with *RASSF1A* methylation in sputum, 3 developed lung cancer within 12 to 14 months of sample collection (62). Collectively, these studies demonstrate that DNA methylation could be a promising biomarker in early lung cancer detection and be useful for population-based screening.

Sputum Detection

Tumor cells or ctDNA is more abudantly present in the most proximate biofluid. Lung cancer detection is more sensitive using respiratory secretions such as sputum (63), bronchoalveolar washings (64), and pleural fluid when compared with plasma. Assessing sputum samples could improve sensitivity of methylation-based approaches to detect lung cancer and augment current screening algorithms. The potential for sputum-based epigenetic testing was demonstrated when Belinsky and colleagues reported that promoter hypermethylation in sputum samples preceded lung cancer development in higher-risk populations (65). In this study, subjects were 25 years or older, smoked greater than or equal to 30 pack-years, and had evidence of obstructive airflow disease by spirometry. Of the 14 genes studied for promoter methylation, 6 were associated with a >50% increased risk in lung cancer, and concomitant methylation of 3 or more genes of the 6 genes was associated with 6.5-fold higher risk of developing lung cancer [95% confidence interval (CI), 1.2–35.5] in sputum samples collected within 18 months of cancer diagnosis. This represents a sensitivity and specificity of 64%.

Hulbert and colleagues reported a sensitive assay to further stratify subjects that had indeterminate nodules on LDCT (63). All subjects were node negative and stage I–IIA. Sputum and plasma were tested using a 6-gene panel to detect methylation of promoter sites using methylation-specific PCR and methylation on beads. The sensitivity and specificity based on the most detected gene were 63% to 86% and 75% to 92%, respectively, in sputum, and 65% to 75% and 74% to 82%, respectively, in plasma. Combination of 3 genes that were most detected resulted in predictably higher sensitivity and specificity of 98% and 71% in sputum and 93% and 62% in plasma, respectively. A similar study by Leng and colleagues also used a methylation assay composed of 8 genes to screen smokers who would identify a "high classifier" subtype to triage people who would benefit from LDCT (66). Using a gene panel on sputum samples from node-negative stage I–IIB lung cancer patients and a control population of cancer-free smokers, they reported a higher odds ratio of 1.6 to 8.9 for methylation of all genes. ROC curves for deducing the diagnostic accuracy of the panel when comparing lung cancer cohort to control cohorts were 82% to 86%. Adding clinical characteristics improved the diagnostic accuracy of the assay. This study utilized large sample size; however, the specificity of an ideal test would need to be higher than the reported 56% when sensitivity is set at 95%.

During a lung bronchoscopy, isotonic saline is flushed into the respiratory tract, and the resultant fluid (bronchoalveolar lavage) is collected for cytological analysis. Liquid biopsies could also be performed on bronchoalveolar lavage (BAL) fluid, albeit this sampling approach would be partly invasive and therefore not useful for population-based screening purposes. When evaluating diagnostic efficiency of methylation panel consisting of *SHOX2* and *RASSF1A*, Zhang and colleagues reported a detection rate of 85.7% using BAL fluid in stage I lung cancer patients, whereas detection rates for CEA and cytology were 10.7% and 46.4%, respectively (67).

Plasma Detection

Methylation-based biomarkers have been extensively studied in plasma and have demonstrated utility in cancer screening, early diagnosis, prognostication, tumor progression, and treatment monitoring. In 2002, a study reported methylation in $p16^{INK4A69}$ in mostly stage I lung cancer patients when compared with control population. In blood, *RASSF1A* and *p16* are most commonly detected in mostly early-stage (0–II) lung cancer patients with reported sensitivity and specificity of 22% to 66% and 57% to 100%, respectively (68–70). Another study of methylation of CpG areas surrounding TSS of *DCLK1* gene in healthy subjects and lung cancer patients (71) reported detection of methylation was 39.3% in stage IIA–IIIA patients, and 56.8% for stages IIIB–IV. Interestingly, methylation was more prevalent in small cell lung cancer than in other cancer subtypes.

In 2010, Ostrow and colleagues demonstrated the use of a multiplex PCR-based panel that evaluated promoter site hypermethylation in 4 tumor suppressor genes (72). They assessed 2 populations: subjects that eventually developed cancerous lesions ($n = 70$) as diagnosed by CT and subjects that developed ground-glass opacities (GGO, $n = 23$). Among the cancerous lesion cohort, 73% had at least 1 gene that was hypermethylated with a specificity of 71%, whereas only 22% of the population with GGO had a hypermethylated gene. There was significant difference in methylation between patients diagnosed with lung cancer and noncancerous lesions ($P = 0.0001$). This study reported that methylation-based panels could discern between cancerous and noncancerous lesions based on CT findings, with reported sensitivity 73% and specificity 71% (AUC 0.643). One of the limitations of the study was no long-term follow-up to determine incidence of lung cancer diagnosis among the control group.

Epi proLung is a liquid biopsy test that includes the genes *PTGER4* and *SHOX2* (73) in a PCR-based approach to detect lung cancer using plasma samples. It is designed as a reflex test for indeterminate pulmonary nodules. For clinical validation, it was tested on 360 samples in the United States and Europe. Of the 360 samples, 152 belonged to patients who were lung cancer positive and the remainder

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belonged to subjects who were cancer negative. Notably among the cancer-positive group, 26 were stage I and 24 were stage IV, and results were not stratified according to tumor stage. At a fixed specificity of 90%, sensitivity for lung cancer was 67%.

Novel approaches

Newer approaches pertaining to methylome sequencing continue to explore new and exciting applications of DNA methylation–based biomarkers. By comparing the CpG cluster patterns of various tissues from The Cancer Genome Atlas (TCGA) database and healthy controls (microarray data) with CpG cluster pattern from plasma of cancer patients, a group was able to predict cancer type and location alongside tumor burden (74). In 2016, EPICUP was released as an assay that could determine origin of tumor with unknown primary with a diagnostic accuracy of 87% (75). The assay is based on array-based whole methylome sequencing of multiple primary tumor types and comparing with the CpG methylation pattern derived from frozen or formalin-fixed, paraffin-embedded tissue samples from known cases of tumor with unknown primary. This approach is mostly limited to labs that can perform sequencing on the Illumina methylation BeadChip platform. Early lung cancer diagnosis remains the holy grail application of methylation-based biomarkers. By performing bisulfite sequencing on lung tumor tissue samples, Liang and colleagues were able to build a diagnostic prediction model to diagnose lung cancer in subjects with indeterminate lung nodules (76). The assay performance was impressive with 75% (55%–90%) sensitivity for stage 1a lung cancer and 85.7% (57%–100%) for stage 1b lung cancer patients.

Methylation-Based Assay Design

For methylation-specific changes to be translated into a good biomarker, a malignancy-specific event should be found ubiquitously in cancer patients and not in subjects with no malignancy. Similarly, a methylation-specific biomarker should only be found in patients with worse prognosis or therapy responders as opposed to nonresponders. If similar regions are methylated in cancer tissue and control tissue, the specificity of the assay will be low. A candidate tumor biomarker will need to fulfill a few characteristics: increased frequency and prevalence of methylated alleles ubiquitously in most/all tumor samples, and absence of background methylation at the same loci in normal tissues. Ideally, these methylation changes at promoter sites should result in differential expression of genes. If methylation occurs at a promoter site of a gene, resulting in repressed expression of that gene, it will biologically make sense to monitor this change as it seems cancer specific and functionally relevant. Once these criteria are fulfilled, a sensitive assay would be needed to detect methylation changes in plasma, as only a subset of tumor-derived DNA will be present in plasma (14).

For methylation-specific biomarkers, location of methylation at promoter site is crucial. For instance, *GSTP1* methylation is noted to be a promising potential biomarker; however, there is a wide array of variability in specificity among studies. One group noted significantly increased specificity when methylation was detected at the 5′ end of the promoter site of the *GSTP1* gene to diagnose hepatocellular carcinoma (97.1% at the 5′ end, 60% at the 3′ end; $P < 0.001$; ref. 77). Another consideration for biomarker design is not all promoter methylation sites are created equal, with significant phenotypic difference from certain changes as opposed to others. A small methylation change at the promoter of the *NMDAR2B* gene just outside of the CpG island was associated with worse prognosis (HR, 3.13; 95% CI, 1.05–9.72; $P \leq 0.006$) in esophageal SCC, whereas methylation changes in more downstream positions in the CpG island were not associated with survival outcomes (78). It is also found that even methylation of a single CpG dinucleotide could affect gene expression. For example, a single CpG dinucleotide is associated with repression of transcription of the *ZAP70* gene, and *ZAP70* gene repression is associated with improved survival in four independent cohorts ($P \leq 0.03$). Another consideration for a candidate biomarker is the number of CpG motifs to cover; optimal length of CpG motif can improve assay specificity. As evident by the examples above, it is important to characterize the exact genomic location of the primer, so that the biomarker performs optimally. It is also important to know the genomic context of the methylation, as promoter sites are usually unmethylated whereas gene exons are usually heavily methylated (79).

To develop a screening diagnostic, a test must be inexpensive, have high sensitivity to reduce false negative, and have high specificity to reduce false positives. False positives can lead to further unnecessary testing and invasive procedures such as bronchoscopy. Liquid biopsy tests can also be used as companion diagnostics in conjunction with current screening practices in an algorithmic manner. In the coming years, epigenetic assays will have a place in the market due to being inexpensive, PCR-based assays with fast turnaround times, or NGS-based assays that can identify new therapeutic targets. NGS-based assays are more expensive but offer new actionable targets when resistance to treatment develops. They will be more feasible in the near future as sequencing costs continue to decrease.

Limitations of Current Studies and Solutions

Despite exponential growth in studies regarding methylation biomarkers for cancer detection, there is currently no clinical assays to aid in lung cancer diagnosis. A major limitation in applying methylation-specific PCR-based approaches for early detection of cancer is the decreased quantity of ctDNA in plasma in patients with early-stage cancers (29). Smaller tumors are reported to have less degree of central necrosis, decreased vascularity, and decreased lymphatic invasion which results in decrease in ctDNA fragments in blood (24). Investigators have reported as low as 2,127 to 8,787 copies of cfDNA, which is equivalent to 7–29 ng from 1 mL of plasma (24, 80). Most of cfDNA is from leukocytes present in blood, and in stage I cancer patients with limited tumor volume, only 1 to 2 copies of ctDNA are found in 1 mL of plasma (29, 81). Methylation-based assays have decreased sensitivity for diagnosing early-stage tumors (82, 83); therefore, their use for screening in a healthy population is challenging. More downstream sample loss (almost >50%) also occurs when samples undergo bisulfite conversion, thereby decreasing assay sensitivity (84). Moreover, methylation-specific quantitative PCR has a higher limit of detection than approaches that are based on targeted deep sequencing (85).

A possible solution is to use NGS- or digital PCR-based approaches which are techniques used for more granular analysis, with a lower limit of detection. Another solution is to limit losses resulting during bisulfite conversion. Methylation on beads was a highly sensitive approach, where investigators reported significant improvement in assay sensitivity by limiting sample losses between reactions (63, 86). Another innovation in the methylome sequencing space is the development of methylation enzyme systems that provide gentler conditions for identification of 5-methyl cytosines. One offering is from NEBNext Enzymatic Methyl-seq (EM-seq) available from New

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England BioLabs and another is from TET-assisted pyridine borane sequencing. These enzymatic systems claim to decrease DNA fragmentation, loss, and bias and as a result provide more amplifiable copies for targeted analysis or NGS.

A potential solution to improve liquid biopsy assay sensitivity is to combine multiple analytes such as ctDNA, ctRNA, and proteins to achieve a higher sensitivity cancer screening assay (87). However, testing concomitantly on the same samples is technically difficult, increases the cost of the assay, and increases the complexity of posttest analysis. Cohen and colleagues developed CancerSEEK that combined serum proteins and ctDNA for early-stage cancer diagnosis; however, the assay sensitivity was 62% with the specificity set at 99% (88). The sensitivity of this approach for stage I lung cancer was however only 43% (20 of 46 patients).

Liquid biopsy tests can also be combined with traditional imaging techniques as complementary diagnostics. Imaging is often more expensive than a simple liquid biopsy test, but does provide tumor location, size, and stage needed for lung cancer diagnosis. Hulbert and colleagues used a sputum and plasma-based methylation assay on subjects with LDCT findings consistent with indeterminate nodules to increase the specificity of screening diagnostics (63). A liquid biopsy test could replace invasive biopsies and procedures after a highly sensitive yet unspecific screening diagnostic (such as LDCT in lung cancer).

Many studies that use methylation-specific PCR report low specificity. One of the reasons for low specificity could be inadequate assay design. Using methylome databases such as TCGA to characterize optimal primer location would result in improved specificity (63, 89). Moreover, methylation of promoter sites that are commonly seen in lung cancer such as *SHOX2* (90), *RASSF1A* (91), *p16* (92), and *NISCH* (93) is also noted to be present in smokers. Therefore, future studies will need to have matched controls that share common demographics. Using bioinformatics for improved analysis could also control for these confounding variables. In the same vein, most of the case–control studies for lung cancer detection report results that are derived from advanced lung cancer cases (stage III/IV) and are not entirely applicable for early cancer diagnosis. In order to evaluate assay sensitivity in early-stage cancers, more studies with early-stage lung cancer need to be performed with matched controls.

Future Directions

DNA-based noninvasive biomarkers have been researched extensively; however, larger multicentered studies are required for clinical validation for translation of epigenetic biomarkers into the clinic. Standardization of DNA methylation protocols is also required for large-scale collaborative efforts to be feasible. As more biofluids are used for assay design, standardization of protocols for processing various biofluids is required. Advancement of whole-genome bisulfite sequencing and microarray-based sequencing techniques will allow for improved profiling of the methylome in cancer patients, healthy subjects, and in plasma. This will improve assay design due to more extensive methylome sequencing data. Innovative techniques such as targeted next-generation sequencing–based panels, digital PCR-based assays, and methylation on beads may improve assay sensitivity and specificity. For lung cancer screening protocols, DNA methylation in plasma/sputum should be used alongside LDCT in large-scale population-based studies to develop novel screening algorithms that decrease the rate of false positives. Because ctDNA consists of somatic mutations and aberrant DNA methylation, combining these two biomarkers would be ideal for improvement in sensitivity and specificity. Harmonization of DNA extraction protocols, methylation sequencing protocols, and transparent access to whole-genome bisulfite sequencing will be needed for collaborative multicenter biomarker validation. The financial strain of noninvasive diagnostics for the healthcare system would need be addressed, because the cost of the test must be considered together with the potential for decreased treatment costs due to earlier cancer diagnosis. Patient advocacy for payer acceptance for these noninvasive diagnostics would also be required. Although DNA methylation-based biomarkers are promising, these hurdles need to be addressed for successful translation of these biomarkers into the clinic.

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References

1. Gierada DS, Pinsky P, Nath H, Chiles C, Duan F, Aberle DR. Projected outcomes using different nodule sizes to define a positive CT lung cancer screening examination. J Natl Cancer Inst 2014;106:dju284.
2. De Koning HJ, Van Der Aalst CM, De Jong PA, Scholten ET, Nackaerts K, Heuvelmans MA, et al. Reduced lung-cancer mortality with volume CT screening in a randomized trial. N Engl J Med 2020;382:503–13.
3. Tammemagi MC, Katki HA, Hocking WG, Church TR, Caporaso N, Kvale PA, et al. Selection criteria for lung-cancer screening. [Erratum appears in N Engl J Med 2013;369:394]. N Engl J Med 2013;368:728–36.
4. National Lung Screening Trial Research Team, Aberle DR, Adams AM, Berg CD, Black WC, Clapp JD, et al. Reduced lung-cancer mortality with low-dose computed tomographic screening. N Engl J Med 2011;365:395–409.
5. Ost D, Fein AM, Feinsilver SH. The solitary pulmonary nodule. N Engl J Med 2003;348:2535–42.
6. Wiener RS, Wiener DC, Gould MK. Risks of transthoracic needle biopsy: how high? Clin Pulm Med 2013;20:29–35.
7. Silvestri GA, Vachani A, Whitney D, Elashoff M, Porta Smith K, Ferguson JS, et al. A bronchial genomic classifier for the diagnostic evaluation of lung cancer. N Engl J Med 2015;373:243–51.
8. Chansky K, Sculier JP, Crowley JJ, Giroux D, Van Meerbeeck J, Goldstraw P. The International Association for the Study of Lung Cancer Staging Project: prognostic factors and pathologic TNM stage in surgically managed non-small cell lung cancer. J Thorac Oncol 2009;4:792–801.
9. Sozzi G, Boeri M, Rossi M, Verri C, Suatoni P, Bravi F, et al. Clinical utility of a plasma-based miRNA signature classifier within computed tomography lung cancer screening: a correlative MILD trial study. J Clin Oncol 2014;32:768–73.
10. Kammer MN, Kussrow AK, Webster RL, Chen H, Hoeksema M, Christenson R, et al. Compensated interferometry measures of CYFRA 21-1 improve diagnosis of lung cancer. ACS Comb Sci 2019;21:465–72.
11. Kossenkov AV, Qureshi R, Dawany NB, Wickramasinghe J, Liu Q, Majumdar RS, et al. A gene expression classifier from whole blood distinguishes benign from malignant lung nodules detected by low-dose CT. Cancer Res 2019;79:263–73.
12. Chapman CJ, Murray A, McElveen JE, Sahin U, Luxemburger U, Türeci O, et al. Autoantibodies in lung cancer: possibilities for early detection and subsequent cure. Thorax 2008;63:228–33.
13. Mandel P, Métais P. Nucleic acids in human blood plasma. C R Acad Sci Paris 1948;142:241–3.

Published OnlineFirst November 4, 2020; DOI: 10.1158/1055-9965.EPI-20-0704

14. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer: a survey. Biochim Biophys Acta Rev Cancer 2007;1775:181–232.
15. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 1989;46:318–22.
16. Sidransky D, Von Eschenbach A, Tsai YC, Jones P, Summerhayes I, Marshall F, et al. Identification of p53 gene mutations in bladder cancers and urine samples. Science 1991;252:706–9.
17. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 2017;14:531–48.
18. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer 2017;17:223–38.
19. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 2015;61:112–23.
20. Alix-Panabières C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discov 2016;6:479–91.
21. Alix-Panabières C, Pantel K. Challenges in circulating tumour cell research. Nat Rev Cancer 2014;14:623–31.
22. Bardelli A, Pantel K. Liquid biopsies, what we do not know (yet). Cancer Cell 2017;31:172–9.
23. Lo YMD, Chan KCA, Sun H, Chen EZ, Jiang P, Lun FM, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med 2010;2:61ra91.
24. Phallen J, Sausen M, Adleff V, Leal A, Hruban C, White J, et al. Direct detection of early-stage cancers using circulating tumor DNA. Sci Transl Med 2017;9:eaan2415.
25. Gai W, Sun K. Epigenetic biomarkers in cell-free DNA and applications in liquid biopsy. Genes 2019;10:32.
26. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. Nat Rev Clin Oncol 2018;15:81–94.
27. Dawson SJ, Tsui DWY, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368:1199–209.
28. Parkinson CA, Gale D, Piskorz AM, Biggs H, Hodgkin C, Addley H, et al. Exploratory analysis of TP53 mutations in circulating tumour DNA as biomarkers of treatment response for patients with relapsed high-grade serous ovarian carcinoma: a retrospective study. PLoS Med 2016;13:e1002198.
29. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224ra24.
30. Lipson EJ, Velculescu VE, Pritchard TS, Sausen M, Pardoll DM, Topalian SL, et al. Circulating tumor DNA analysis as a real-time method for monitoring tumor burden in melanoma patients undergoing treatment with immune checkpoint blockade. J Immunother Cancer 2014;2:42.
31. Oshiro C, Kagara N, Naoi Y, Shimoda M, Shimomura A, Maruyama N, et al. PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. Breast Cancer Res Treat 2015;150:299–307.
32. Newman AM, Bratman S V, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20:548–54.
33. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget 2015;6:42008–18.
34. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012;4:136ra68.
35. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985–990.
36. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med 2015;21:795–801.
37. Chabon JJ, Simmons AD, Lovejoy AF, Esfahani MS, Newman AM, Haringsma HJ, et al. Circulating tumour DNA profiling reveals heterogeneity of EGFR inhibitor resistance mechanisms in lung cancer patients. Nat Commun 2016;7:11815.
38. McDonald BR, Contente-Cuomo T, Sammut SJ, Odenheimer-Bergman A, Ernst B, Perdigones N, et al. Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer. Sci Transl Med 2019;11:eaax7392.
39. Tie J, Cohen JD, Wang Y, Li L, Christie M, Simons K, et al. Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. Gut 2019;68:663–71.
40. Montani F, Marzi MJ, Dezi F, Dama E, Carletti RM, Bonizzi G, et al. MiR-Test: a blood test for lung cancer early detection. J Natl Cancer Inst 2015;107:djv063.
41. Baylin SB. The cancer epigenome: its origins, contributions to tumorigenesis, and translational implications. Proc Am Thorac Soc 2012;9:64–5.
42. Baylin SB, Jones PA. Epigenetic determinants of cancer. Cold Spring Harb Perspect Biol 2016;8:a019505.
43. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev 2002;16:6–21.
44. Sato T, Arai E, Kohno T, Takahashi Y, Miyata S, Tsuta K, et al. Epigenetic clustering of lung adenocarcinomas based on DNA methylation profiles in adjacent lung tissue: its correlation with smoking history and chronic obstructive pulmonary disease. Int J Cancer 2014;135:319–34.
45. Selamat SA, Galler JS, Joshi AD, Fyfe MN, Campan M, Siegmund KD, et al. DNA methylation changes in atypical adenomatous hyperplasia, adenocarcinoma in situ, and lung adenocarcinoma. PLoS One 2011;6:e21443.
46. Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, et al. DNA methylation markers and early recurrence in stage I lung cancer. N Engl J Med 2008;358:1118–28.
47. Sato T, Arai E, Kohno T, Tsuta K, Watanabe S, Soejima K, et al. DNA methylation profiles at precancerous stages associated with recurrence of lung adenocarcinoma. PLoS One 2013;8:e59444.
48. Sandoval J, Mendez-Gonzalez J, Nadal E, Chen G, Carmona FJ, Sayols S, et al. A prognostic DNA methylation signature for stage I non-small-cell lung cancer. J Clin Oncol 2013;31:4140–7.
49. Robles AI, Arai E, Mathé EA, Okayama H, Schetter AJ, Brown D, et al. An integrated prognostic classifier for stage I lung adenocarcinoma based on mRNA, microRNA, and DNA methylation biomarkers. J Thorac Oncol 2015;10:1037–48.
50. Walter K, Holcomb T, Januario T, Du P, Evangelista M, Kartha N, et al. DNA methylation profiling defines clinically relevant biological subsets of non-small cell lung cancer. Clin Cancer Res 2012;18:2360–73.
51. Shinjo K, Okamoto Y, An B, Yokoyama T, Takeuchi I, Fujii M, et al. Integrated analysis of genetic and epigenetic alterations reveals CpG island methylator phenotype associated with distinct clinical characters of lung adenocarcinoma. Carcinogenesis 2012;33:1277–85.
52. Collisson EA, Campbell JD, Brooks AN, Berger AH, Lee W, Chmielecki J, et al. Comprehensive molecular profiling of lung adenocarcinoma: the cancer genome atlas research network. Nature 2014;511:543–50.
53. Hawes SE, Stern JE, Feng Q, Wiens LW, Rasey JS, Lu H, et al. DNA hypermethylation of tumors from non-small cell lung cancer (NSCLC) patients is associated with gender and histologic type. Lung Cancer 2010;69:172–9.
54. Carvalho RH, Hou J, Haberle V, Aerts J, Grosveld F, Lenhard B, et al. Genome-wide DNA methylation analysis identifies novel methylated genes in non-small-cell lung carcinomas. J Thorac Oncol 2013;8:562–73.
55. Holdenrieder S, Stieber P, Bodenmüller H, Busch M, Fertig G, Fürst H, et al. Nucleosomes in serum of patients with benign and malignant diseases. Int J Cancer 2001;95:114–20.
56. Ward TH, Cummings J, Dean E, Greystoke A, Hou JM, Backen A, et al. Biomarkers of apoptosis. Br J Cancer 2008;99:841–6.
57. Fahrner JA, Eguchi S, Herman JG, Baylin SB. Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. Cancer Res 2002;62:7213–8.
58. Palmisano WA, Divine KK, Saccomanno G, Gilliland FD, Baylin SB, Herman JG, et al. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res 2000;60:5954–8.
59. Kersting M, Friedl C, Kraus A, Behn M, Pankow W, Schuermann M. Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers. J Clin Oncol 2000;18:3221–9.
60. Destro A, Bianchi P, Alloisio M, Laghi L, Di Gioia S, Malesci A, et al. K-ras and p16INK4A alterations in sputum of NSCLC patients and in heavy asymptomatic chronic smokers. Lung Cancer 2004;44:23–32.
61. Zöchbauer-Müller S, Lam S, Toyooka S, Virmani AK, Toyooka KO, Seidl S, et al. Aberrant methylation of multiple genes in the upper aerodigestive tract epithelium of heavy smokers. Int J Cancer 2003;107:612–6.
62. Honorio S, Agathanggelou A, Schuermann M, Pankow W, Viacava P, Maher ER, et al. Detection of RASSF1A aberrant promoter hypermethylation in sputum

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from chronic smokers and ductal carcinoma in situ from breast cancer patients. Oncogene 2003;22:147–50.
63. Hulbert A, Jusue-Torres I, Stark A, Chen C, Rodgers K, Lee B, et al. Personalized medicine and imaging early detection of lung cancer using DNA promoter hypermethylation in plasma and sputum. Clin Cancer Res 2017;23:1998–2005.
64. Um SW, Kim HK, Kim Y, Lee BB, Kim D, Han J, et al. Bronchial biopsy specimen as a surrogate for DNA methylation analysis in inoperable lung cancer. Clin Epigenetics 2017;9:131.
65. Belinsky SA, Liechty KC, Gentry FD, Wolf HJ, Rogers J, Vu K, et al. Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. Cancer Res 2006;66:3338–44.
66. Leng S, Wu G, Klinge DM, Thomas CL, Casas E, Picchi MA, et al. Gene methylation biomarkers in sputum as a classifier for lung cancer risk. Oncotarget 2017;8:63978–85.
67. Zhang C, Yu W, Wang L, Zhao M, Guo Q, Lv S, et al. DNA methylation analysis of the SHOX2 and RASSF1A panel in bronchoalveolar lavage fluid for lung cancer diagnosis. J Cancer 2017;8:3585–91.
68. Bearzatto A, Conte D, Frattini M, Zaffaroni N, Andriani F, Balestra D, et al. p16INK4A hypermethylation detected by fluorescent methylation-specific PCR in plasmas from non-small cell lung cancer. Clin Cancer Res 2002;8:3782–7.
69. Ponomaryova AA, Rykova EY, Cherdyntseva NV, Skvortsova TE, Dobrodeev AY, Zav'yalov AA, et al. Potentialities of aberrantly methylated circulating DNA for diagnostics and post-treatment follow-up of lung cancer patients. Lung Cancer 2013;81:397–403.
70. Nunes SP, Moreira-Barbosa C, Salta S, Palma de Sousa S, Pousa I, Oliveira J, et al. Cell-free DNA methylation of selected genes allows for early detection of the major cancers in women. Cancers 2018;10:357.
71. Powrózek T, Krawczyk P, Nicoś M, Kuźnar-Kamińska B, Batura-Gabryel H, Milanowski J. Methylation of the DCLK1 promoter region in circulating free DNA and its prognostic value in lung cancer patients. Clin Transl Oncol 2016;18:398–404.
72. Ostrow KL, Hoque MO, Loyo M, Brait M, Greenberg A, Siegfried JM, et al. Molecular analysis of plasma DNA for the early detection of lung cancer by quantitative methylation-specific PCR. Clin Cancer Res 2010;16:3463–72.
73. Weiss G, Schlegel A, Kottwitz D, König T, Tetzner R. Validation of the SHOX2/PTGER4 DNA methylation marker panel for plasma-based discrimination between patients with malignant and nonmalignant lung disease. J Thorac Oncol 2017;12:77–84.
74. Kang S, Li Q, Chen Q, Zhou Y, Park S, Lee G, et al. CancerLocator: non-invasive cancer diagnosis and tissue-of-origin prediction using methylation profiles of cell-free DNA. Genome Biol 2017;18:53.
75. Moran S, Martínez-Cardús A, Sayols S, Musulén E, Balañá C, Estival-Gonzalez A, et al. Epigenetic profiling to classify cancer of unknown primary: a multicentre, retrospective analysis. Lancet Oncol 2016;17:1386–95.
76. Liang W, Zhao Y, Huang W, Gao Y, Xu W, Tao J, et al. Non-invasive diagnosis of early-stage lung cancer using high-throughput targeted DNA methylation sequencing of circulating tumor DNA (ctDNA). Theranostics 2019;9:2056–70.
77. Jain S, Chen S, Chang KC, Lin YJ, Hu CT, Boldbaatar B, et al. Impact of the location of CpG methylation within the GSTP1 gene on its specificity as a DNA marker for hepatocellular carcinoma. PLoS One 2012;7:e35789.
78. Kim MS, Yamashita K, Chae YK, Tokumaru Y, Chang X, Zahurak M, et al. A promoter methylation pattern in the N-methyl-D-aspartate receptor 2B gene predicts poor prognosis in esophageal squamous cell carcinoma. Clin Cancer Res 2007;13:6658–65.
79. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484–92.
80. Ørntoft MBW. Review of blood-based colorectal cancer screening: how far are circulating cell-free DNA methylation markers from clinical implementation? Clin Colorectal Cancer 2018;17:e415–e33.
81. Lui YYN, Chik KW, Chiu RWK, Ho CY, Lam CWK, Dennis Lo YM. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem 2002;48:421–7.
82. Church TR, Wandell M, Lofton-Day C, Mongin SJ, Burger M, Payne SR, et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. Gut 2014;63:317–25.
83. Pedersen SK, Baker RT, McEvoy A, Murray DH, Thomas M, Molloy PL, et al. A two-gene blood test for methylated DNA sensitive for colorectal cancer. PLoS One 2015;10:e0125041.
84. Ørntoft MBW, Jensen SØ, Hansen TB, Bramsen JB, Andersen CL. Comparative analysis of 12 different kits for bisulfite conversion of circulating cell-free DNA. Epigenetics 2017;12:626–36.
85. Chaudhuri AA, Binkley MS, Osmundson EC, Alizadeh AA, Diehn M. Predicting radiotherapy responses and treatment outcomes through analysis of circulating tumor DNA. Semin Radiat Oncol 2015;25:305–12.
86. Bailey VJ, Zhang Y, Keeley BP, Yin C, Pelosky KL, Brock M, et al. Single-tube analysis of DNA methylation with silica superparamagnetic beads. Clin Chem 2010;56:1022–5.
87. Elazezy M, Joosse SA. Techniques of using circulating tumor DNA as a liquid biopsy component in cancer management. Comput Struct Biotechnol J 2018;16:370–8.
88. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science 2018;359:926–30.
89. Koch A, Joosten SC, Feng Z, de Ruijter TC, Draht MX, Melotte V, et al. Analysis of DNA methylation in cancer: location revisited. Nat Rev Clin Oncol 2018;15:459–66.
90. Biesterfeld S, Ilse P, Pomjanski N, Wrobel C, Schramm M. Analysis of SHOX2 methylation as an aid to cytology in lung cancer diagnosis. Cancer Genom Proteom 2014;11:251–8.
91. Hsu HS, Chen TP, Hung CH, Wen CK, Lin RK, Lee HC, et al. Characterization of a multiple epigenetic marker panel for lung cancer detection and risk assessment in plasma. Cancer 2007;110:2019–26.
92. Belinsky SA, Klinge DM, Dekker JD, Smith MW, Bocklage TJ, Gilliland FD, et al. Gene promoter methylation in plasma and sputum increases with lung cancer risk. Clin Cancer Res 2005;11:6505–11.
93. Ostrow K, Michailidi C, Guerrero-Preston R, Hoque MO, Greenberg A, Rom W, et al. Cigarette smoke induces methylation of the tumor suppressor gene NISCH. Epigenetics 2013;8:383–8.

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